Molecular enhancement of porcine cardiac chronotropy

J M Edelberg, D T Huang, M E Josephson, R D Rosenberg

Abstract

Objective—To test the potential of gene transfer approaches to enhance cardiac chronotropy in a porcine system as a model of the human heart.

Methods—Plasmids encoding either the human β_2 adrenergic receptor or control constructs were injected into the right atria of native Yorkshire pig hearts. Percutaneous electrophysiological recording catheters equipped with 33 gauge circular injection needles were positioned in the mid-lateral right atrium. At the site of the earliest atrial potential the circular injection needles were rotated into the myocardium and the β_2 adrenergic receptor (n = 6) or control plasmid constructs (n = 5) were injected.

Results—Injection of the β_2 adrenergic receptor construct significantly enhanced chronotropy compared with control injections. The average (SD) heart rate of the pigs was 108 (16) beats/min before injection. Two days after injection with control plasmids the heart rate was 127 (25) beats/min (NS compared with preinjection rates). After injection with plasmid encoding the β_2 adrenergic receptor the heart rate increased by 50% to 163 (33) beats/min ($p < 0.05$ compared with preinjection and postinjection control rates).

Conclusions—The present studies showed in a large animal model that local targeting of gene expression may be a feasible modality to regulate cardiac pacemaking activity. In addition, these investigations provide an experimental basis for developing future clinical gene transfer approaches to upregulate heart rate and modulate cardiac conduction.

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Keywords: sinus node; adrenergic receptor; chronotropic agents; conduction system; gene therapy

Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215, USA
J M Edelberg
D T Huang
M E Josephson

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
R D Rosenberg

Correspondence to:
Dr Edelberg
jme2002@mail.med.cornell.edu

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Cardiac chronotropic incompetence is associated with increased morbidity and mortality.[1–3] Permanent chronotropic disorders caused by sinus node diseases include inappropriate sinus bradycardia, sinus arrest, and sinus exit block.[4 5] These diseases are not confined to the aged, being occasionally diagnosed in young adults,[6 7] and may have a familial component.[8–10] The pathology of the disease is diverse. Anatomical studies have shown the presence of fibrosis and sclerodermal infiltrates in the sinus node associated with advanced age.[11 12] Ischaemic and infectious causes have also been linked to chronotropic incompetence.[13–15] The treatment of these disorders is limited, with only a minority of the transient disorders amenable to medical treatment.[16] The majority of the causes of chronotropic incompetence require the implantation of an electronic pacemaker, either temporarily or permanently.[17] Future treatments for chronotropic incompetence may be based on therapeutics that can specifically enhance the pacemaker potential of the endogenous cardiac tissue and potentially diminish the necessity for exogenous electronic devices.

Recent advances in biomedical research suggest that gene therapy may be used in the treatment of cardiovascular disease.[18 19] Cardiac pacemaking offers a unique target in gene therapy as modulation of the spontaneous depolarisations in a selected set of cells may enhance heart rate. Recently we reported the development of a murine gene transfer model to test the effects of candidate genes on heart rate.[20] In this model constructs encoding the human β_2 adrenergic receptor were used under in vitro, ex vivo, and in vivo conditions to enhance selectively murine cardiac chronotropy, suggesting a potential role for this approach in future treatments of cardiac pacemaking disorders.

The translation into clinical treatment requires that this strategy be further tested and optimised in large animal models. The Yorkshire pig model was used in the present study based on its anatomical and physiological similarity to the human cardiovascular system.[21] These studies offered the opportunity to develop a transvenous catheter delivery gene transfer approach to enhance cardiac chronotropy, which may be adapted for future human investigations.

Methods

PLASMID CONSTRUCTS

A 2.25 kb *Sal*I-*Bam*HI fragment, the human β_2 adrenergic receptor complementary DNA (cDNA), was ligated into a *Sal*I-*Bam*HI site 3' to the β actin promoter in a pBR322 vector to generate pBR322-β actin promoter-β_2 adrenergic receptor. In similar fashion, the bacterial β galactosidase gene was ligated to the β actin promoter in a pBR322 vector and served as a control vector. The plasmid construct encoding the humanised green fluorescent protein[22] with a cytomegalovirus promoter element was purchased from Clontech (Palo Alto, California, USA) and served as a co-injection vector. The recombinant DNA injection solutions (200 µl; 100 µg DNA/ml; 5:1 M/M β_2 adrenergic receptor or β galactosidase/green fluorescent protein)

were prepared with in an injection vehicle of phosphate buffered saline (PBS) with 20% sucrose and 2% Evans blue.

INJECTION CATHETER

The electrophysiological recording catheter was custom designed and manufactured by Medtronic, Inc (Minneapolis, Minnesota, USA). The polyurethane coated catheter was 7 French gauge and was supported with an 8 French sheath. The distal end of the catheter was terminated with a three and a half turn 33 gauge corkscrew shaped needle allowing it to impale tissues securely to record local intracardiac electrograms. The proximal end of the catheter was terminated with a luer lock injection port allowing it to accept standard sized syringes.

NATIVE HEART DNA INJECTION

Female Yorkshire pigs weighing 15–20 kg were initially anaesthetised with intramuscular ketamine (10 g/kg) and intubated. The animals were then given 2% isoflurane and ventilated with a large animal ventilator (Hallowell model 2000, Pittsfield, MA, USA). Heart rate, blood pressure, and arterial oxygen saturation were monitored for the duration of the procedure. By sterile technique, the right femoral vein was exposed and cannulated, and an 8 French sheath was inserted. Under fluoroscopic guidance the electrophysiological injection catheter was introduced and advanced to the right atrium. Simultaneous six lead surface and intracardiac electrocardiograms were recorded with a multichannel recorder (EVR PPG Biomedical, Pittsburgh, PA, USA). The A-P interval (ms), cycle length (ms), PR interval (ms), and P wave axis (°) were measured. At the site of the earliest A wave the injection needle was rotated 270° into the atrial myocardium. The recombinant DNA construct (200 μl) controls (n = 5) or β_2 adrenergic receptor samples (n = 6) were then injected into the atrial myocardium in a randomised double blinded protocol. The catheter was then disengaged and removed from the animal. The animals were observed for an additional 10 min and monitored for complications. The vascular sheath was then removed, the vein was sutured, and the incision site was closed. Anaesthesia was then discontinued. After they regained spontaneous respirations the animals were placed in individual pens. The animals were monitored hourly for the next 3 h and then daily until termination of the experiments, 48–96 h after injection.

SERIAL SURFACE ECGS AND ANALYSIS

Serial surface ECGs were recorded daily for all animals for the duration of the study. The pigs were anaesthetised with ketamine as above. Simultaneous six lead surface ECGs were recorded. The cycle length, PR interval, and P wave axis were measured (mean (SD)). Significance was determined by a Student's *t* test analysis between average preinjection and postinjection heart rates with the control and β_2 adrenergic receptor plasmid injections, and between the percentage change in paired preinjection and postinjection heart rates with the control and β_2 adrenergic receptor plasmid injections.

β_2 ADRENERGIC RECEPTOR IMMUNOSTAINING

At the termination of the experiments, the animals were sacrificed and the hearts explanted. The injection sites were identified by Evans blue and harvested unfixed for cryosectioning and immunostaining. The samples were cut in to 10 μm sections and fixed with cold methanol for 10 minutes. The sections were then washed with PBS and blocked with 10% normal serum in PBS for 20 minutes. Samples were then incubated with a rabbit antihuman β_2 adrenergic receptor polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, California, USA; 1.0 μg/ml) for one hour in a humid chamber at 25°C. The samples were washed with PBS three times and then incubated with a secondary donkey antirabbit Cy3 polyclonal antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) at a 1:1000 dilution in PBS with 1% bovine serum albumin for one hour in a humid chamber at room temperature. The samples were washed with PBS three times and mounted with 90% glycerol in PBS. Green fluorescent protein expression was identified by using epifluorescence filters (excitation 405 nm/emission 490 nm). Immunostaining for the human β_2 adrenergic receptor was identified by using epifluorescence filters (excitation 488 nm/emission 540 nm).

Results

The animals were anaesthetised and intubated, and venous access was obtained as described above. The injection catheter was advanced to the right lateral atrium under fluoroscopic guidance (fig 1A). Simultaneous surface and intracardiac ECGs were recorded. The catheter was positioned at the site of the earliest atrial activity, as fig 1B shows. The atrial potential at the injection sight was similar in the pigs injected with the control (14 (10) ms) and the β_2 adrenergic receptor encoding constructs (12 (10) ms). In addition, both the average PR interval and the P wave axis on the surface ECG were similar in the groups before injection (table 1). All the animals tolerated the procedure well.

Serial surface ECGs recorded from the pigs after construct injection showed that the average PR interval and P wave axis were similar to the measurements before injection (table 1). The heart rate increased in the hearts injected with the β_2 adrenergic receptor plasmid compared to hearts with the control injections (fig 2A). The average heart rate of the pigs was 108 (16) beats/min before injection. Two days after injection with control plasmids the heart rate was 127 (25) beats/min ($p > 0.3$ compared with preinjection). After injection with plasmid encoding the β_2 adrenergic receptor the heart rate increased to 163 (33) beats/min ($p < 0.05$ compared with preinjection), an approximately 50% increase in heart rate ($p < 0.01$ compared with the change in postinjection control heart rate) (fig 2B). All

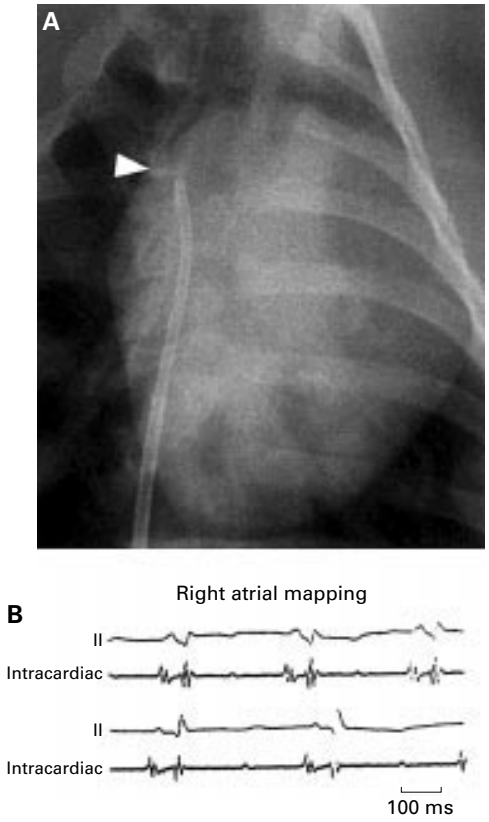

Figure 1 Cine of electrophysiological recording and injection catheter (arrow head) during injection of complementary DNA in to the porcine lateral right atrium (A). Two examples are shown of surface and intracardiac ECGs recorded before injection into the right atrium (B).

Table 1 Electrocardiographic measurements

	Baseline		*48 h postinjection*	
	Control	*β_2AR*	*Control*	*β_2AR*
P wave axis (°)	65 (7)	56 (20)	60 (20)	62 (15)
PR interval (ms)	85 (10)	92 (9)	86 (8)	86 (9)

β_2AR, β_2 adrenergic receptor.

animals survived until the termination of the experiments.

Injection of the cDNA constructs led to the expression of the encoded genes. Sections of the right atrial tissue at the site of injection showed the presence of green fluorescent protein (fig 2C,D). Immunostaining of the injection site sections showed colocalisation of the human β_2 adrenergic receptor in the hearts co-injected with the β_2 adrenergic receptor encoding constructs (fig 2D) but not in those injected with control constructs (fig 2C).

Discussion

The present experiments were directed at developing in vivo gene transfer techniques to identify and study genes that can be used to upregulate heart rate selectively and alter cardiac rhythm in the intact heart in a large animal model. Our goal is eventually to develop a molecular or cellular based approach to the treatment of cardiac chronotropic and conduction disorders. We have focused our initial efforts on a direct porcine cardiac gene transfer

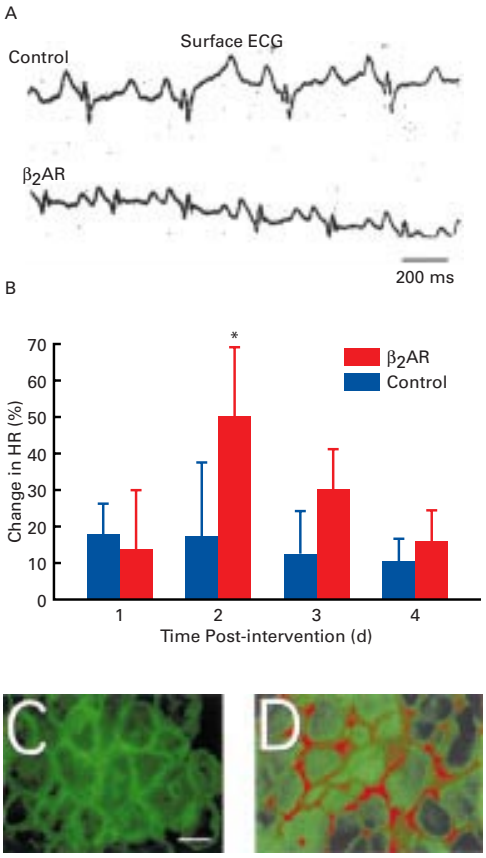

Figure 2 (A) Representative surface ECGs recorded 48 hours after injection of either control constructs or constructs encoding β_2 adrenergic receptor. (B) The average percentage change in heart rate (HR) after injection of control construct or β_2 adrenergic receptor (β_2 AR) encoding plasmids ($p < 0.01$ versus control). Dual fluorescence micrographs of sections of right atrial tissue co-injected with plasmids encoding green fluorescent protein and control constructs (C) and with the human β_2 adrenergic receptor (D). Green fluorescent protein was visualised directly (green), and the human β_2 adrenergic receptor was detected by immunostaining (red). Bar 32 μm.*

system to translate our recently developed murine cDNA chronotropic test system[20] into a clinically applicable endovascular therapeutic approach.

The transvenous delivery of the cDNA constructs was electrophysiologically guided. Constructs were injected into the area of the sinus node in an attempt to enhance the chronotropy of the intact cardiac conduction system. The mappings of the perisinus node injection sites were in agreement with previous characterisations of the porcine sinus node.[23] Delivery of the cDNA encoding the human β_2 adrenergic receptor resulted in a significant increase in the heart rate compared with control injections. The non-significant change in the control injection heart rate may have been caused by the level of anaesthesia or the intubation of the animals at baseline, as has previously been shown in porcine anaesthesia studies.[24 25]

The β_2 adrenergic receptor injection enhancement of chronotropy was not associated with altered characteristics of atrial conduction, with no significant changes in either the PR interval or the P wave axis. These data suggest that the increased heart rate is mediated by

an increased rate of sinus node depolarisations. Alternatively, the enhanced heart rate may arise from a perisinus nodal atrial focus that conducts normally through the atrium. Future approaches will target atrial sites removed from the sinus node, including the left atrium, to test the potential of gene transfer to establish independent pacemaking foci.

The transience of the increase in porcine heart was expected given the use of plasmid cDNA constructs. Previous research has shown that injection of plasmid into the left ventricular myocardium may result in prolonged construct expression (> 1–2 months)[26 27] compared with the 2–3 days observed in the murine[20] and the present porcine right atrial injections. The limited duration seen in the present study is likely multifactorial. Previous studies have shown significant differences in the expression of plasmid constructs injected into different muscle groups.[28 29] These regional variations may have an anatomical basis as a result of technical injection limitations as well as variable DNA diffusion rates in various tissues.[28] Additional factors, including atrial T tubule density,[30 31] may also account for the relatively short period of plasmid mediated enhancement of cardiac chronotropy, and different vector strategies will be required to sustain the increased heart rates. To this end, advances in adenoviral and adenoviral associated viral technologies offer efficient approaches for more extended expression of constructs in cardiac tissues.[32–34] Moreover, cardiac myocyte transplantation approaches[35 36] may allow for the engraftment of a more permanent genetically engineered tissue to modulate the cardiac conduction system.

In summary, these studies showed that the basal rate of the heart can be enhanced by local intravascular delivery of exogenous genes. These investigations may provide a foundation for developing novel therapeutics for cardiac chronotropic disorders, such as sick sinus syndrome.

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